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MICROBIAL-MALATHION INTERACTION IN ARTIFICIAL SALT-MARSH ECOSYSTEMS

Al W. Bourquin

Environmental Protection Agency

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The disappearance of malathion in the salt-marsh environment is influenced by both chemical and biological degradation; however, at temperatures below 26 C and salinities below 20 °/oo, chemical mechanisms appear to be of less importance than biological degradation.

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Microbial-Malathion Interaction in Artificial Salt-Marsh Ecosystems

Effect and Degradation

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MICROBIAL-MALATHION INTERACTION IN ARTIFICIAL SALT-MARSH ECOSYSTEMS

Effect and Degradation

by

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Malathion is rapidly degraded in vitro by salt-marsh bacteria to malathion-monocarboxylic acid, malathion-dicarboxylic acid and various phosphothionates as a result of carboxyesterase cleavage. In addition, some expected phosphatase activity produces desmethyl-malathion, phosphothionates, 4-carbon dicarboxylic acids, and corresponding ethyl esters.

In a simulated salt-marsh environment, malathion is degraded by the indigenous bacterial community. Numbers of bacteria capable of degrading malathion in the presence of additional nutrients increase in the sediments with increasing frequency of application and in the water column with the increasing level of treatment. Numbers of bacteria which degrade malathion as a sole carbon source are linked to the level of treatment in sediments and the frequency of treatment in the water column; however, these bacteria do not appear to play a significant role in the dissipation of malathion. I believe that frequency of treatment, increases numbers of malathion co-metabolizing bacteria which catalyze a more rapid dissipation of the compound, which results in fewer sole carbon degraders.

The disappearance of malathion in the salt-marsh environment is influenced by both chemical and biological degradation; however, at temperatures below 26 C and salinities below 20 °/oo, chemical mechanisms appear to be of less importance than biological degradation.

This report was submitted in fulfillment of ROAP 10AKC, Task 006.by the Gulf Breeze Environmental Research Laboratory under the sponsorship of the Environmental Protection Agency. Work was completed as of December 1973.

CONTENTS

Section		Page
I	CONCLUSIONS	1
II	INTRODUCTION	2
III	MATERIALS AND METHODS	
	Materials	7
	Experimental Methods	7
	Analytical Methods	7
	Statistical Analysis	11
		13
IV	RESULTS	
	Physico-Chemical Degradation	14
	Microbiological Degradation	14
	Isolation and Identification of Microbial Metabolites	14
	Artificial Ecosystem Studies	18
		29
V	DISCUSSION	35
VI	REFERENCES	38
VII	ABBREVIATIONS AND SYMBOLS	41

LIST OF FIGURES

Figure		Page
1	Enzymatic cleavage of malathion	6
2	Range Point Salt-Marsh on Santa Rosa Island, Florida	8
3	Schematic for extraction of malathion metabolites	12
4	Effect of salinity and temperature on malathion stability	15
5	Bacterial degradation of malathion in seawater	19
6	Thin-layer chromatogram of microbial and chemical degradation products	21
7	Infrared spectral tracings of (a) malathion and TLC spot 1, (b) malathion-half-ester and TLC spot 2	22
8	Infrared spectral tracing of malaoxon with representative adsorption bands indicated	23
9	Infrared spectral tracings of (a) malathion-dicarboxylic acid and TLC spot 3, (b) K-desmethyl-malathion and TLC spot 4	25
10	Infrared spectral tracings of (a) TLC spot 5, (b) TLC spot 6	26
11	Infrared spectral tracings of (a) TLC spot 7, (b) TLC spot 8	27
12	Infrared spectral tracings of (a) diethyl maleate and TLC spot 9 (b) TLC spot 10	28
13	Bacteria from water column of artificial salt-marsh ecosystem treated with malathion	30
14	Bacteria from sediments of artificial salt-marsh ecosystems treated with malathion	31
15	Bacteria from water column of artificial salt-marsh ecosystems treated with mirex	33
16	Bacteria from sediments of artificial salt-marsh ecosystems treated with mirex	34

LIST OF TABLES

Table		Page
1	Estimated U. S. Insecticide Production, 1971	2
2	Structural Formulae of Malathion, Metabolites and By-Products	4
3	Microbial Growth on Malathion Plus Nutrients	14
4	Microbial Degradation of Malathion	17
5	Cell-Medium Distribution ^{14}C from Malathion	17
6	Radiometric and Chemical Analyses of Microbial Growth on ^{14}C-Malthion	18
7	R_f Values (Thin-Layer Chromatography) for Malathion and Metabolites	20
8	Malathion Degradation Products	36

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SECTION I
CONCLUSIONS

Malathion is rapidly degraded *in vitro* by salt-marsh bacteria to malathion-monocarboxylic acid, malathion-dicarboxylic acid and various phosphothionates as a result of carboxyesterase cleavage. In addition, some expected phosphatase activity produces desmethyl-malathion, phosphomono- or -dithionates, and various 4-carbon dicarboxylic acids, as well as corresponding ethyl esters.

In a simulated salt-marsh environment, malathion is degraded by the indigenous bacterial community. Numbers of bacteria capable of degrading malathion in the presence of additional nutrients increase in the sediments with increasing frequency of application and in the water column with the increasing level of application. Numbers of bacteria which degrade malathion as a sole carbon source appear to be linked to the level of malathion treatment in sediments and the frequency of malathion treatment in the water column. Malathion sole-carbon-degrading bacteria do not appear to play a significant role in the dissipation of malathion, comprising only about 10% of the portion of bacteria which degrades malathion. It is believed that due to increased frequency of treatment, increased numbers of malathion co-metabolizing bacteria catalyze a more rapid dissipation of the compound, resulting in less selection of the sole carbon degraders.

The disappearence of malathion in the salt-marsh environment is influenced by both chemical and biological degradation. Chemical hydrolysis increases with increasing temperature and salinity, but at temperatures below 26 C and salinities below 20 $^{o}/_{oo}$, these mechanisms are of lesser importance than biological degradation.

SECTION II
INTRODUCTION

Estuarine environments are composed of highly complex communities of organisms, some of which are inseparably linked to coastal marshlands. The most productive parts of the estuary are the intertidal and adjacent shallow-water zones (23). Coastal nekton frequently use estuaries as nursery grounds where larval through adolescent growth stages can take advantage of the protection and abundant food. Because early life stages of many important commercial and sport fisheries depend on estuaries, it is important economically to protect these habitats. These saline marshlands are also prime breeding sites for mosquitoes. Recently, massive mosquito control programs have been established to control the adult mosquito in municipal areas and, in many instances, effective treatment has necessitated insecticide application on or near marshes which serve as nursery grounds for a variety of marine species (7). Concern for possible hazards to non-target species in the salt-marsh has prompted studies to determine the fate and effects of these chemical toxicants.

Malathion, S-(1,2-Dicarbethoxyethyl)-O, O-dimethyl dithiophosphate, is an organophosphate insecticide used extensively to control adult mosquitoes. It was the single most widely used insecticide in the United States in 1971, estimated annual production being 1.36×10^7 kg (30 million lbs., Table 1). In a single operation in 1971, the U. S. Air Force applied 15.3×10^4 ℓ (40,335 gal.) of technical grade malathion (active ingredient unknown) to 2,016,060 acres in counties

Table 1. ESTIMATED U. S. INSECTICIDE PRODUCTION VOLUME, 1971[a]

Chemical group	Number compounds	Production (Mill. lbs. A.I.)
Chlorinated hydrocarbons	13	158
Carbamate	5	64
Organophosphate	23[b]	148[b]
Others	51	23
Total, all synthetic organic insecticides, miticides, nematocides		393

[a]From U.S. EPA Pesticide Study Series, 1971 (26)
[b]Malathion = 24% of total organophosphate production

along the coast of the Gulf of Mexico to control mosquitoes carrying Venezulan Equine Encephalomyelitis virus.

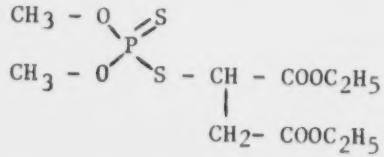
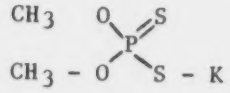
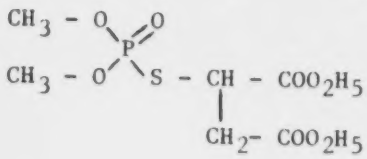
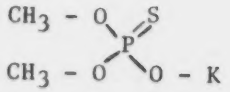
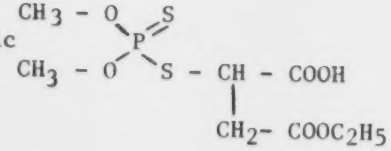
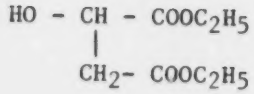
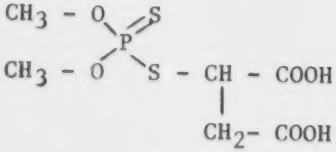
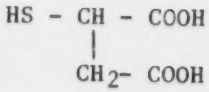
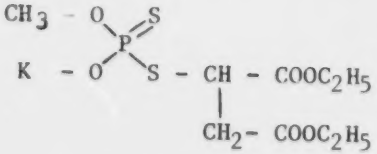
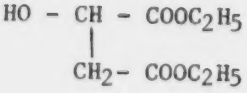
Malathion has been reported toxic to shrimp (Penaeus duorarum) at a concentration of 0.5 ppm (EC_{50}, 48 hours) (5). Also, chronic exposures of spot (Leiostomus xanthurus) to malathion (10 µg/ℓ flowing seawater for 26 weeks) significantly reduced brain acetylcholinesterase activity (14). Field studies in marsh embayments of the Texas coast tested the effects of aerial application of malathion in a concentration (6 oz/acre) normally used for mosquito control on juvenile commercial shrimp, Penaeus azteus (Ives) and Penaeus setiferus (Linn) (7). Shrimp suffered mortalities ranging from 14 to 80 percent, whereas shrimp from control areas suffered no deaths attributable to the pesticide. Reported malathion residues were as high as 2.39 ppm in dead shrimp and 2.61 ppm in live shrimp and malathion was found at most test sites. Malathion concentrations in water samples from test sites ranged from 0.00 to 3.20 ppm (7). In another field test (24), no apparent adverse effects of malathion on resident or confined animals were reported when malathion was applied either as a thermal fog or as ultra-low-volume mist (ULV) spray on a Florida salt-marsh.

The mode of action of malathion in insects, as well as other animals, is inhibition of acetylcholinesterase systems (8, 13). Blockage of this enzyme system in mammals results in respiratory failure and in insects, death probably results from the organisms's inability to move or to feed due to loss of voluntary muscular coordination (13).

Malathion is an organophosphate insecticide (see Table 2 for structural formula) synthesized primarily by the addition of dimethyl-dithiophosphoric acid to the diethyl-ester of maleic acid (21). It contains 36.35% carbon, 5.80% hydrogen, 9.38% phosphorous, 19.41% sulfur and 29.06% oxygen and has a molecular weight of 330.36. Malathion has a solubility of 145 mg/ℓ in distilled water and is miscible with many organic solvents (3).

Like most organophosphates, malathion is relatively unstable in the environment. Degradation of malathion occurs non-enzymatically, chiefly by hydrolysis (10), and enzymatically, by way of two systems--phosphatases and carboxyesterases singly or in combination (Figure 1). The two modes of degradation may be complementary in the breakdown of the insecticide in soil (25). Seventy-two hours following application in an estuary, malathion could not be detected at temperatures above 15.6 C (60F); hence, was reported to be completely degraded; however, no mechanism of dissipation was proposed (9). Immediately after application to a salt-marsh in another study, a high concentration of

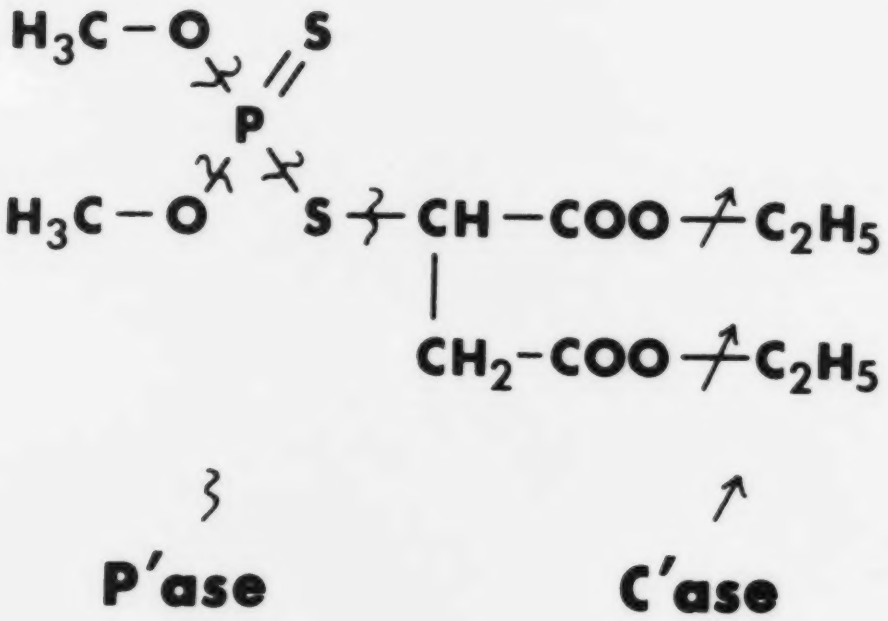
Table 2. STRUCTURAL FORMULAE OF MALATHION, METABOLITES, AND BY-PRODUCTS

Name	Chemical structure	Name	Chemical structure
Malathion		Dimethyl phosphorodithioate, potassium salt	
Malaoxon		Dimethyl phosphorothioate, potassium salt	
Malathion half-ester (monocarboxylic acid, MCA)		Diethyl-maleate	
Malathion dicarboxylic acid (DCA)		Mercaptosuccinate	
O-Desmethyl malathion, potassium salt (KDM)		Diethyl-succinate	

malathion was observed, but progressively lower concentrations were observed at 24-, 33-, and 48-hours after application (7). Similarly, in another study in which malathion was applied to a marsh (24), insecticide concentrations in marsh water immediately after fogging and ULV application were 5.2 and 0.49 ppb, respectively, but only trace amounts (0.1 - 0.3 ppb) persisted as long as one day. This rapid dissipation of parent compound is probably a consequence of both chemical and biological degradation. Although malathion is readily degraded chemically, it also serves as a substrate for microbial degradation (18, 28), and several species of soil fungi and bacteria capable of attaching malathion and some of its breakdown products have been isolated (20). Similarly, in a study designed to assess the relative importance of chemical, as opposed to microbiological, degradation of malathion in several Mississippi soils, Walker and Stojanovic (1973a) found microbial degradation to be the chief mechanism of insecticide dissipation in all soils tested.

Despite numerous studies of the fate and effects of malathion in estuarine salt-marsh ecosystems or in soil systems, little information is known of its microbiological fate or its effects on estuarine bacteria and fungi. The study reported here had three primary objectives: (1) to isolate by enrichment culture bacteria capable of readily metabolizing malathion, (2) to isolate and identify the major metabolites of malathion resulting from this microbial degradation, and (3) to determine fate of malathion in a simulated salt-marsh environment and its effect on bacteria.

Figure 1. Enzymatic cleavage of malathion. Products of phosphatase (P'ase) and carboxyesterase (C'ase). Products of malathion cleavage are given in Table 2.



SECTION III
MATERIALS AND METHODS

MATERIALS

Malathion, malaoxon, and the carboxylic acid products were obtained from American Cyanamid Co., Princeton, N. J. ^{14}C-methoxy-malathion was received from Mallinckrodt, St. Louis, MO. Analytical-grade standards for chromatography were obtained from EPA, Pesticides Reference Standards Section, Chemistry Branch, Washington, D.C; standards for IR analyses were synthesized by the EPA, Southeast Environmental Research Laboratory, Athens, GA.

Microbiological media used were purchased from Baltimore Biological Laboratory, Baltimore, Md. All organic solvents used for pesticide extraction were of nanograde quality (Mallinckrodt Chemical Works, St. Louis, MO) and others were of spectroanalyzed grade (Fisher Scientific Co., Fair Lawn, N. J.).

EXPERIMENTAL METHODS

Preparation of malathion-utilizing bacteria- To obtain bacteria for study, sediment and water samples were collected from a salt-marsh on Santa Rosa Island, Florida (Fig. 2). The area was considered free of malathion contamination (not previously sprayed by Escambia County Mosquito Control Department), the water temperature was 28°C, and the salinity was 20 °/oo (parts per thousand). Duplicate sets of both water (10 m ℓ) and sediment (10 g) samples were inoculated into 250 mℓ Erlenmeyer flasks that contained 90 mℓ Zobell's Marine 2216 Broth (1) or aged seawater (Rila Marine Mix, Teaneck, N. J. of 20 °/oo salinity and malathion was added at the rate of 100 mg/ℓ in 1.0 mℓ acetone. The 250 mℓ Erlenmeyer flasks were incubated on a rotary shaker at 28°C for 30 days. Ten-milligram aliquots of malathion in 0.5 mℓ acetone were added to the flasks every 7 days to maintain a high pesticide concentration and adequately large carbon source for malathion-utilizing microbes. Samples from each flask were streaked on seawater agar and Marine 2216 agar enriched with malathion (100 mg/liter). Colonies were picked from these plates for pesticide-utilization tests (See Microbial Degradation Studies).

Physico-Chemical Degradation- The physico-chemical stability of malathion was tested in sterile seawater solutions. The ranges of light, temperature, and salinity were selected to cover only those employed in microbial degradation tests. Flasks that contained 50 mℓ sterile Rila Sea-Salts adjusted with distilled water to salinities of 0, 10, 20 and 30 °/oo were inoculated with sterile

Figure 2. Santa Rosa Island, near Gulf Breeze, Florida. Arrow indicates Range Point salt-marsh.

malathion (filter-sterilized, 0.2μ) at a concentration of 1.0 mg/ℓ. Duplicate flasks of each salinity were incubated at 20 C or 28 C in continuous darkness (wrapped in aluminum foil) under 6,000 lux illumination from Growlux® fluorescent tubes with alternating 12-h periods of light and darkness. Duplicate flasks of each kind were removed and extracted every two days. Malathion concentration was determined as described in Gas-Liquid Chromatography (see below).

Microbial Degradation Studies- Selected isolates from enrichment cultures were screened qualitatively for biodegradation of malathion. Two-tenths milliliter of an 18-hour culture grown in 10 mℓ of marine 2216 broth was inoculated into 10 mℓ of sterile sea-salts medium containing malathion (100 μg/mℓ) and incubated for 5 days at 28 C on a rotary shaker. Cells and medium were extracted without separation with 10 mℓ petroleum ether in the incubation tube and analyzed for residual insecticide by gas-liquid chromatography. Ability of each culture to degrade malathion was then compared with that of the control (sterile seasalt medium, malathion, but no cells). For quantitative determinations, isolates which exhibited greatest ability to degrade malathion were tested by inoculating approximately 10^5 cells, washed with sterile sea-salts solution from an 18-hour agar-slant culture, into sea-salts medium containing malathion (46 μg/mℓ), with or without 0.2% peptone. The cultures were incubated for 10 days at 28 C on a rotary shaker before extracting malathion for analysis by gas-liquid chromatography.

Bacteria shown by the quantitative tests to be most efficient in the utilization of malathion as a sole-carbon source were tested for the ability to incorporate ^{14}C derived from ^{14}C-methoxymalathion. Cultures grown in marine broth were diluted 1:100 into fresh seawater medium containing 200 μg/mℓ malathion and 0.1 μCi-^{14}C in Biometer flasks (Bellco, Inc.). Reaction vessels were incubated for 14 days at 28 C and $^{14}CO_2$ sampled daily for $^{14}CO_2$ evolution. Radioactive CO_2 was recovered by adding 1.0 mℓ alcoholic-hyamine solution (1M, Packard Instruments) to the side-arm of the Biometer flask, incubating 5 minutes, and removing the $^{14}CO_2$-hyamine solution with a syringe. The hyamine solution was added to toluene-scintillation cocktail (10 mℓ) and analyzed on a liquid-scintillation counter.

After 14 days, the culture medium was treated with 0.5 mℓ of 0.2% trichloracetic acid (final pH = 2) and immediately extracted twice with petroleum ether. The solvent phase was washed with 20 mℓ 0.5 M potassium phosphate buffer, pH 7. All fractions were assayed radiometrically to determine extent of malathion breakdown. The ether phase contained malathion and some degradation products. The aqueous fractions at pH 2 contained the carboxyesterase products and those at pH 7 contained phosphatase and other hydrolysis products (20). The ether phase and ether:acetone (1:1) extracts of the pH 2 water were analyzed further by gas-liquid chromatography.

The actual rates of malathion degradation were determined by incubation in seawater medium containing 46 µg/mℓ malathion and sampling at two-day intervals for malathion residues. Sterile peptone was added to cultures showing no greater reduction in malathion than control cultures (malathion-seawater but no cells). These cultures were monitored for malathion degradation after an additional four days of incubation.

Several bacterial isolates that represented both sole-carbon degraders and co-metabolizers were inoculated into 10 mℓ sea-sal-0.2% peptone medium containing 0.1 µCi-^{14}C-malathion (46 µg/mℓ). After 10 days incubation, residual malathion was extracted with petroleum ether. The cells were removed by centrifugation at 12,000 xg for 10 minutes, washed with 10 mℓ sterile seawater, dried for 24 hours at 85 C, and analyzed for ^{14}C-activity by liquid scintillation counting. The cell-free supernate was analyzed for soluble degradation products by assaying for residual ^{14}C-activity.

Cultures grown on marine 2216 broth and marine broth plus 200 µg malathion/mℓ were analyzed for contribution of malathion to cell mass. After 10 days incubation, the cells were separated by centrifugation and transferred to tared aluminum weighing dishes. The cells were dried for 16 h at 110 C and weighed.

To determine malathion metabolites, Pseudomonas sp. 45 was incubated in seawater medium and Pseudomonas sp. 8 was incubated in seawater medium with 2% peptone, both containing 100 µg/mℓ malathion. (In earlier screening studies, these organisms degraded malathion as a sole carbon source and as a co-substrate.) Cultures were incubated 10 days at 28 C in the dark. Cells and medium were then separated by centrifugation and the cell-free medium was extracted, with petroleum ether to remove malathion. The aqueous fraction was (see Fig. 3) extracted again with petroleum ether: acetone (1:1) and with diethyl ether. The concentrated extract was analyzed for malathion and metabolites by thin-layer chromatography.

Preparation of Laboratory Ecosystems- Sediment and water collected from a salt marsh on Santa Rosa Island, near Gulf Breeze, Florida (Figure 2) was transported to the laboratory for initial planting of a laboratory salt-marsh ecosystem. Sediment (sand) from the Range Point salt-marsh area was sieved through a No. 7 mesh screen to remove larger particles and facilitate sampling procedures. Sieved sand (7.62 cm deep) was then placed into acetone-cleaned battery jars (16 x 16 x 26 cm) and 6.5 liters of marsh water added. A Pasteur pipette was fixed about 2/3 distance down in the water column, and air was slowly bubbled through to prevent anaerobiosis or surface slick formation. The laboratory micro-ecosystem was allowed to settle for 72 hours before sampling. Water temperature and salinity were maintained at 28 C (± 1 C) and 20 °/oo (± 2 °/oo) for the duration of the experiment. The artificial ecosystems were maintained under a 12 hour cycling light-dark system (6,000 lux).

Malathion was added in 0.5 mℓ acetone to the water surface of two battery jars at 420 g/ha (field application rate) and 4.2 kg/ha (10X field rate). Since previous investigation indicated no significant effects on indigenous microflora resulted from the addition of 0.5 m acetone to similar experimental ecosystems, no acetone controls were included in this experiment. The artificial ecosystems were treated every 10 days during two 30-day experiments. This cycle was chosen to simulate a mosquito-control program.

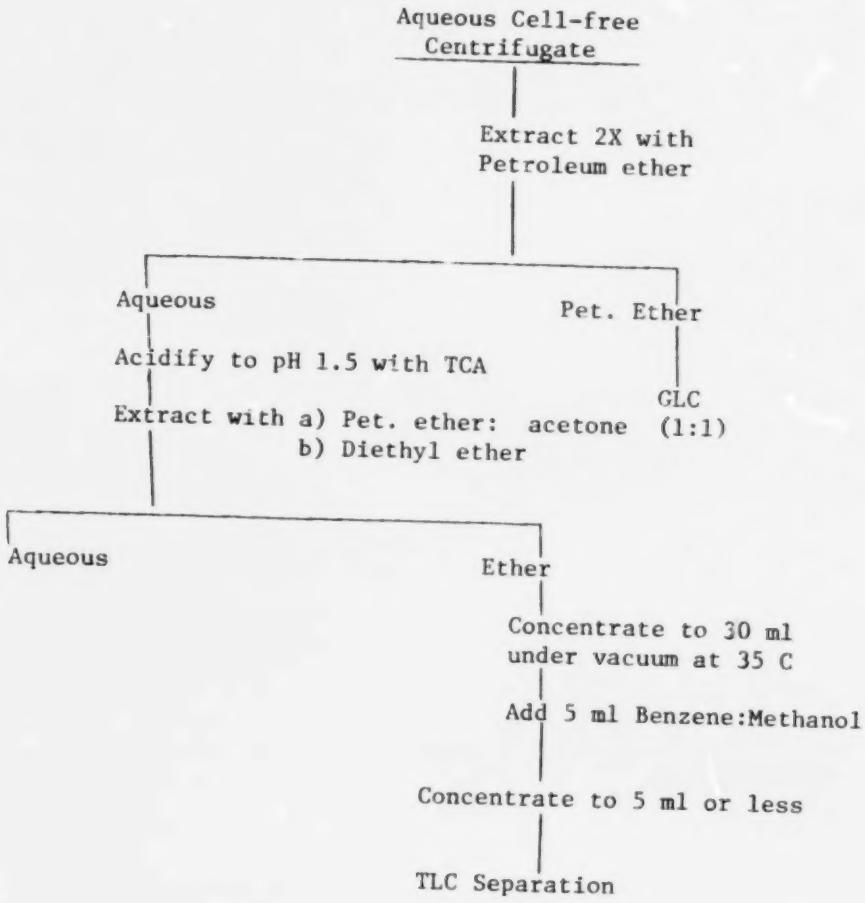
After the initial 72-hour settling period, water and sediment samples were assayed microbiologically to insure that microbial numbers had returned to nominal environmental levels. Once stabilization was ascertained, malathion was added and the test period begun. Ten-milliliter water samples, taken from the middle of the water column, were added to 90 mℓ sterile seawater blanks and diluted appropriately for plate counts. Ten-gram (wet-weight) sediment samples were taken with a large-bore sterile pipette and diluted for plate counts. Sediment and water samples were taken immediately before treatment, at "0" time (immediately after treatment) and at 1, 3, 7 and 10 day intervals thereafter. Also, pH of the water was checked and samples were collected for analysis of residual malathion and total organic carbon.

The effects of malathion treatment on numbers of heterotrophic bacteria, malathion co-metabolizing bacteria, and chitinoclastic bacteria were determined by plating appropriately diluted water and sediment samples (yields 30-100 colonies/plate) on marine agar, on marine agar plus 50 µg/mℓ malathion, and on 1% chitin-agar (15), respectively. After 3 days incubation at 28 C, the heterotrophic plates were checked for hydrolysis of starch, lipid, and casein by the replica-plating technique. This technique allows the screening of a large number of colonies for numerous biochemical activities with a minimum of transfers. A selected plate, the master plate, is pressed onto a sterile velveteen pad stretched over a post. The pad is then used to replicate the master plate colonies onto various media to check growth or biochemical activity. The master plates were chosen from appropriate dilutions such that they contained 50-100 colonies per plate. Replica plates were incubated at 28 C for 4 days, at which time amylase, lipase, and casein producers were enumerated. Media were prepared according to the methods of Colwell & Wiebe (6).

ANALYTICAL METHODS

Gas-Liquid Chromatography (GLC)- Residual malathion and malathion breakdown products extracted from the various physio-chemical and microbial degradation studies were quantitated by gas-liquid chromatographic methods. Two instruments, a Varian-Aerograph Model 2100 equipped with a tritium source electron-capture detector and a Tracor Model MT-220 with a Melpar flame-photometric detector, were employed. For the electron-capture GLC determinations, two columns, one containing 2% OV-101 on

Figure 3. Extraction schematic for malathion metabolites.



100/120 mesh Gas Chrom Q and the other column 0.75% OV-17 and 0.85% OV-210 on 100/120 Gas Chrom Q, were employed. Both columns were 0.64 cm x 1.8 m. Column, detector, and inlet temperatures were 200, 250, and 250 C, respectively. The carrier gas was nitrogen, used at a flow rate of 25 mℓ/min. For the flame photometric analyses, the detector was operated in the phosphorus mode. A 0.32 cm x 1.8 m column containing 2% OV-101 on 80/100 mesh Gas-Chrom Q was used for all analyses. Respective column, detector (ignited), and injector temperatures were 180, 160, and 220 C, and gas flow rates for O_2, air, H_2, and N_2 (carrier) were 20, 50, 200, and 60 mℓ/min, respectively.

Amyl derivatives of malathion degradation products were prepared for gas chromatography analyses by previously described methods (23). All samples were quantified by comparing the peak height with those standards of known concentration.

Thin-Layer Chromatography (TLC)- Extracts from biodegradation studies were separated on 250 μ-thick Silica-gel H, 20 cm X 20cm, thin-layer glass plates prepared by Quanta/Gram™ (A. H. Thomas Co., Phil., PA.). Plates were spotted with 10-20 μℓ of concentrated extract and developed in the appropriate solvents. For qualitative analyses, two-dimensional chromatography was employed using the following solvents: (1) Benzene:Hexane:Acetic Acid (40:40:20) and (2) Hexane:Acetic Acid:Ether (75:15:10) (18). After allowing the plates to dry, they were sprayed with 0.5% (wt/vol) N, 2, 6-Trichloro-p-benzoquinoneimine (TCQ, Eastman Kodak Co., Rochester, N. Y.) freshly prepared in nanograde acetone. The plates were then developed at 110 C for 10 minutes (10). Spots representing malathion and its degradation products appear as dark, reddish-pink on a light background.

Infrared Spectroscopy (IR)- To prepare metabolites for infrared spectral analyses, 1-2 mℓ of the concentrated acetone extract was streaked on a TLC plate and developed with benzene:glacial acetic acid (4:1) in one direction. After allowing the plate to dry, it was covered with another glass plate that allowed only about two cm of the TLC plate to be exposed, then sprayed with TCQ (29). Areas corresponding to metabolite bands were scraped from the plate and extracted with 50 mℓ acetone. This extract was concentrated to 1-2 mℓ under vacuum at 35 C and an appropriate aliquot added to dried potassium bromide (Harshaw) for analysis on a Perkin-Elmer Model 621 Grating Infrared Spectrophotometer. Whenever appropriate, the samples were analyzed by operating the instrument in a 5X expanded ordinate scale. Spectral tracings for malathion degradation products were compared to standards supplied by American Cyanamid Co. and EPA's Southeast Environmental Research Laboratory.

STATISTICAL ANALYSIS

Data obtained from the artificial ecosystem studies were treated by analysis of variance to determine existence of significant differences (1) between control and experimental cultures over a 30-day treatment period, (2) during a single treatment period (every 10 days) and (3) at each treatment level.

SECTION IV
RESULTS

PHYSICO-CHEMICAL DEGRADATION

As shown in Figure 4, the hydrolytic degradation of malathion increases with increasing salinities. The data are presented as ranges and averages of residual malathion detected at various lengths of incubation. Four replications were employed for each salinity treatment, one light- and one dark- incubated sample from each temperature. Although malathion degradation in distilled water was observed to a limited extent, the rate of degradation was much slower than in seawater. No effect of light was observed under the test conditions, but temperatures above 26.7 C (80 F) increased the rate of degradation. Breakdown products from the chemical degradation of malathion were identified as malathion monocarboxylic acid (detected at two days) and malathion dicarboxylic acid (detected at seven days) by flame photometric gas chromatography by comparing their retention times with those of standards. Although malaoxon was detected, it did not increase in quantity over that found in the stock malathion.

MICROBIOLOGICAL DEGRADATION

Indirect evidence for malathion utilization as a carbon source by bacterial species was shown by growth studies using marine broth 2216 plus malathion. Dry-weight determinations were performed as indicated in the methods section of this report and growth stimulation by malathion was apparent from the greater cell mass produced (Table 3). However, the increased cell mass could not be due to malathion carbon alone, since the total carbon available was only about 8 mg. No explanation for the increased cell mass can be given at this time.

Table 3. MICROBIAL GROWTH ON MALATHION PLUS NUTRIENTS.

	Dry weights (mg) after 10 days incubation		
Culture number	Inoculum	Marine[a]	Marine + Malathion[b]
1	40	127	134
12	32	115	108
44	58	145	150
45	35	125	165
47	65	155	185
Mixture[c]	70	250	200

[a] Marine 2216 broth. [b] Marine 2216 broth plus 200 µg Malathion/ml medium.
[c] Five isolates in equal proportions.

Estuarine bacteria isolated from Range Point salt-marsh sediment by malathion- enrichment techniques were tested for biodegradative ability by incubating a washed- cell suspension containing approximately 10^5 cells/mℓ in sea-salts medium containing 46 µg/mℓ malathion and in the same medium with 2% peptone as a supplementary energy source. Residues analyzed by GLC after 10-days incubation are expressed in Table 4 as

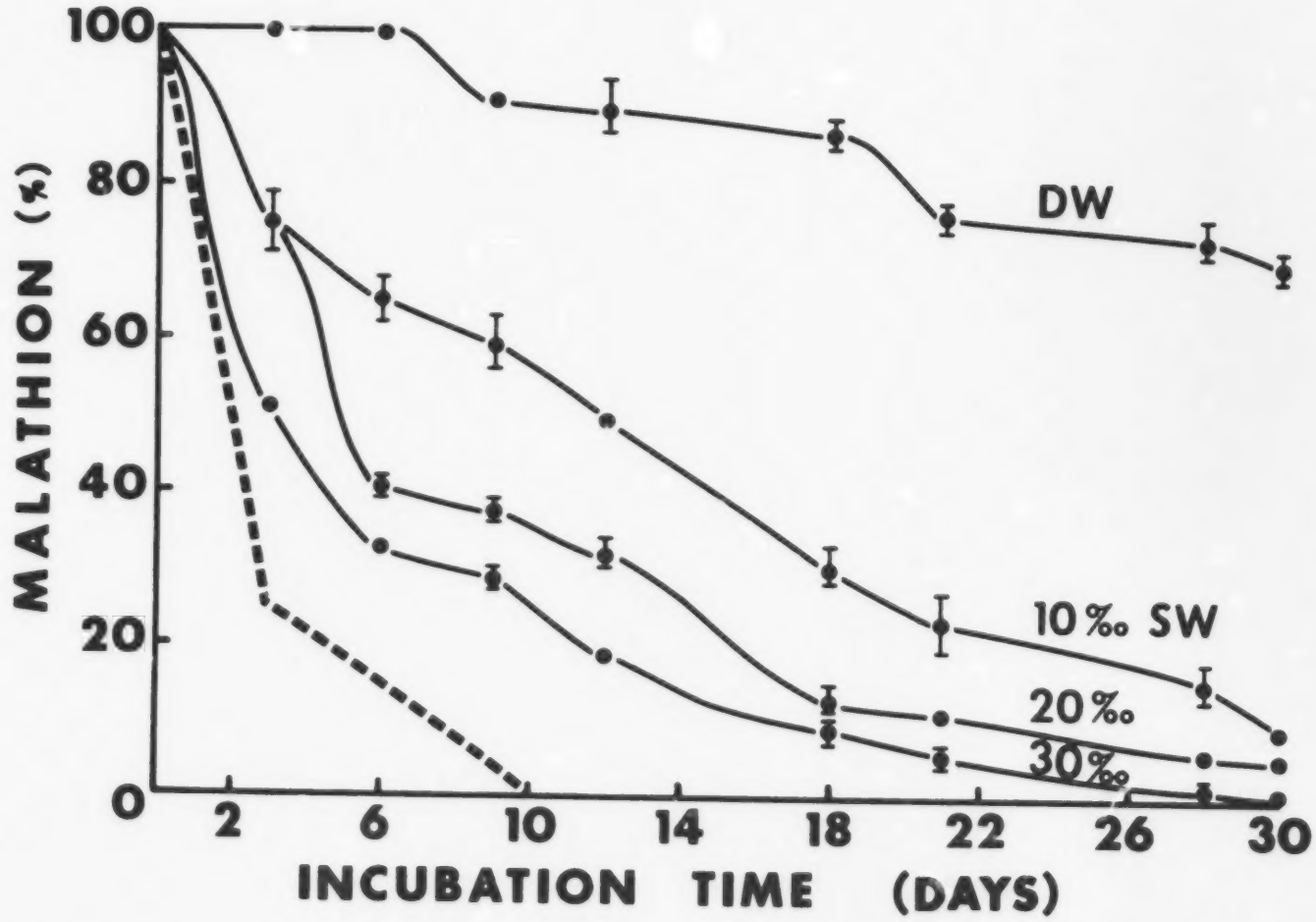


Figure 4. Effect of salinity and temperature on malathion stability. Malathion 1 µg/mℓ added in acetone to sterile seawater at varied salinities, 0 °/oo distilled water. Dashed line represents microbial degradation.

percentage of added malathion degraded. Controls consisted of malathion media without added cells. Most isolates were not able to use malathion effectively as a sole carbon source. Forty-percent of the isolates tested showed 50% or greater utilization of malathion. However, in the presence of peptone, malathion was rapidly degraded by all isolates tested.

Bacterial cultures were grown on ^{14}C-malathion with peptone and analyzed by liquid-scintillation counting to determine the extent of malathion-carbon incorporation in the microbial cell-mass. Most of the activity was associated with the cell fractions; however, 8-28% remained in the supernate (Table 5). This fraction probably represented water-soluble metabolites of malathion. For assessment of radioactive malathion metabolites, spent medium from ^{14}C-malathion incubation as a sole carbon source was assayed for carboxyesterase phosphatase products by differential extraction and liquid scintillation counting, and confirmed by chemical analysis (Table 6). The carboxyesterase products, MCA and DCA, were identified by GLC retention times and quantitated. There is good agreement between chemical residue analysis and isotopic analysis for malathion. In most cases, MCA is the predominate degradation product produced by biological as well as chemical reactions, as indicated in the control data. The data indicate a greater portion of phosphatase products due to biological breakdown (cultures 44, 45, 47), with lesser production of similar products due to chemical breakdown (control). The "mixture" indicated in Table 6 represents equal proportions of the aforementioned cultures. The degrading activity of the mixture was greater than the individual cultures, as evidenced by the lower recovery and higher DCA concentration. The microbial systems shown here have an effective carboxyesterase system that causes rapid breakdown of malathion to the acids, with a delayed demethylation reaction to produce demethyl-malathion. Some microbial systems apparently catalyze demethylation earlier, resulting in fast release of CO_2 from the malathion molecule. Data from cultures 1 through 12, not shown here, indicated that cultures 1, 4 and 9 affected the release of $^{14}CO_2$ within 2 days, whereas others required incubation for 7 days before appreciable release of $^{14}CO_2$ was detected. In the sterile control, 20% of the ^{14}C-methoxymalathion label was released as $^{14}CO_2$ after 10 days incubation.

Table 4. MICROBIAL DEGRADATION OF MALATHION.

Culture number	Morphology & gram reaction	Degraded[a] as S.C.S. (%)	Degraded[b] with 0.2% Peptone (%)
1	(-) short rod	48	91
2	(-) coccoid rod	2	81
3	(-) medium rod	32	83
4	(-) medium rod	66	100
5	(+) short rod	36	94
6	(-) medium rod	2	82
7	(-) medium rod	1	83
8	(-) short rod	28	90
9	fungus	1	77
10	fungus	50	91
11	(-) slender rod	24	92
12	(-) medium rod	71	100
44	(+) slender rod	90	73
45	(-) short rod	72	100
47	(-) large ovoid rod	87	100

[a]After 10 days incubation as sole carbon source. [b]After 5 days incubation with 0.2% Peptone.

Table 5. CELL-MEDIUM DISTRIBUTION OF ^{14}C FROM MALATHION[a]

Culture number	Percentage of total radioactivity	
	Wet cells	Supernatant
1	76	24
2	92	8
3	87	13
4	72	28
5	89	11
6	90	10
7	81	19
8	93	7
9	80	20
10	83	17
11	87	13
12	91	9

[a]Incubation medium was seawater medium containing (46 µg/mℓ) ^{14}C-malathion plus 0.2% peptone in all cases.

Table 6. RADIOMETRIC AND CHEMICAL ANALYSES OF MICROBIAL GROWTH ON ^{14}C - MALATHION.

Radiometric analysis - Percentage of total radioactivity remaining in expended medium

Culture No.	Malathion	Products of Carboxyesterase	Products of Phosphatase
44	25.7	36.8	37.5
45	8.3	48.2	43.5
47	6.5	61.3	32.2
Mixture	7.4	87.2	5.4
Control[a]	10.5	82.4[a]	7.1[a]

Residue Analyses- flame photometric GLC analysis - ppm

Culture No.	Recovery (ppm) Malathion	MCA	DCA	Total	Percentage recovery
44	19	85	40	144	72
45	75	159	8	172	86
47	75	80	23	108	54
Mixture	75	11	55	75	38
Control[a]	9	168[a]	7[a]	184	92

[a]Sterile medium plus ^{14}C - malathion, products are due to chemical breakdown.

Figure 5 shows the results of four different cultures incubated with seawater-malathion media. The results are plotted as percentage malathion remaining at time of sampling. Parent compound remaining in control flasks at each sampling time (i.e., percentage remaining after chemical degradation) shown as percentage of the original concentration, are presented in parentheses above each sampling day. Two cultures (No. 1 and 12) readily utilized malathion, whereas two cultures (No. 6 and 9) incubated for 10 days showed little degradation of the molecule beyond that of chemical degradation. However, the latter cultures readily metabolized the insecticide when 0.2% peptone was added. Microbial degradation, with or without additional nutrient, is dependent upon the type of microbe utilized, but is faster than chemical degradation, as observed in these cultures.

ISOLATION AND IDENTIFICATION OF MICROBIAL METABOLITES

Spent sea-water malathion medium from cultures 8 and 45, with and without added nutrient, was extracted, concentrated, and assayed by thin-layer chromatography to separate and tentatively identify degradation products.

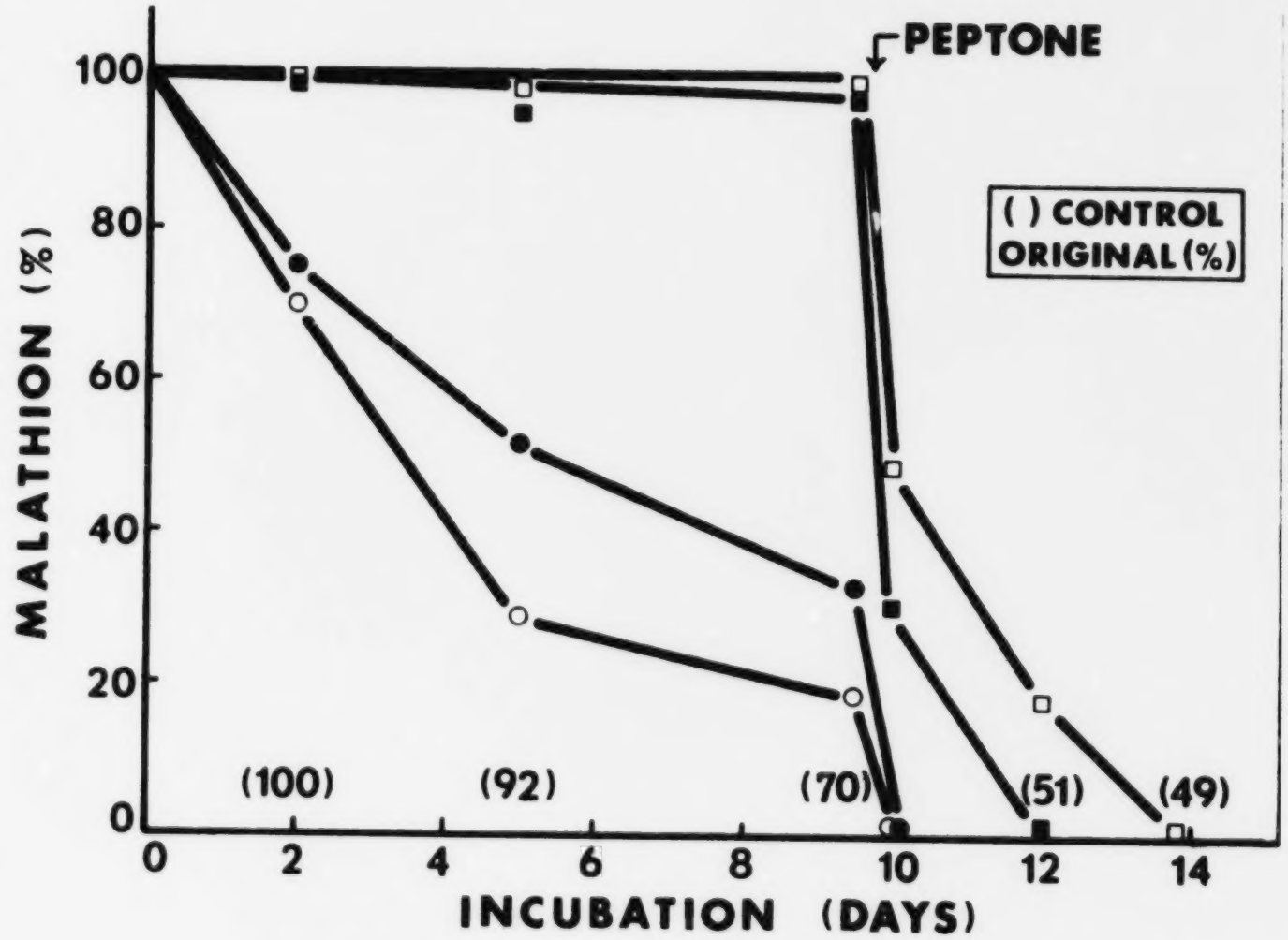


Figure 5. Bacterial degradation of malathion in seawater.

Good separation of the malathion and its metabolites was achieved on a two-dimensional TLC. Figure 6 shows typical chromatograms from microbial degradation and control media (malathion but no cells). Four compounds - the two carboxylic acids, malathion, and K-desmethyl-malathion were tentatively identified by comparison with known compounds. The values shown in Table 7, match reference materials well. Other metabolites, indicated by numbers 5, 6, 7 and 8, were probably phosphothionates, judging from published R_f values (18), but no standards were available for comparison. With the exception of spot number 8, most of the latter compounds were not present on the control plate and, therefore, are probably microbial metabolites. No differences in metabolites could be detected between the two cultures since all compounds visible in culture 8 extracts were also visible in those of culture No. 45.

Table 7. TLC - R_f VALUES FOR MALATHION AND METABOLITES.

Compound	Solvent[a]	Solvent[b]
Malathion	0.93	0.95
Malaoxon	0.80	0.77
KDM	0.63	0.23
MCA	0.71	0.68
DCA	0.28	0.31

[a]Hexane : Acetic Ac : DEE (2 : 2 : 1)
[b]Benzene : Acetic Ac : DEE (75 : 15 : 10)

Figures 7 and 8 show the infrared spectral tracings of analytical grade malathion and malaoxon with major adsorption bands indicated. Explanations of infrared spectral analysis of malathion and metabolites are taken from Walker and Stojanovic, (29), and Jones (17). Adsorption peaks at 2960 and 1450 reciprocal centrimeters (cm^{-1}) represent asymmetrical C-H stretches, whereas those at 2940 and 1375 cm^{-1} represent symmetrical C-H stretches. Bands at 2550 cm^{-1} represent S-H stretch bonds. A strong band at 1730 cm^{-1} represents a C=O, whereas a band 1000-1200 cm^{-1} indicates C-O (1170 cm^{-1}). Methyl and C=C groups adsorb at 1380 and 1640 cm^{-1}, respectively. The band at 1010 cm^{-1} indicates P-O-C bonding and C-C stretch bonds are indicated at 1100 cm^{-1}. The band at 655 cm^{-1} represents P=S bond and the weak peaks at 515 and 490 cm^{-1} possibly represent P-S.

The spectral tracing of TLC spot 1, when compared with that of malathion in Figure 7, appears to be the same compound. Therefore, it was concluded

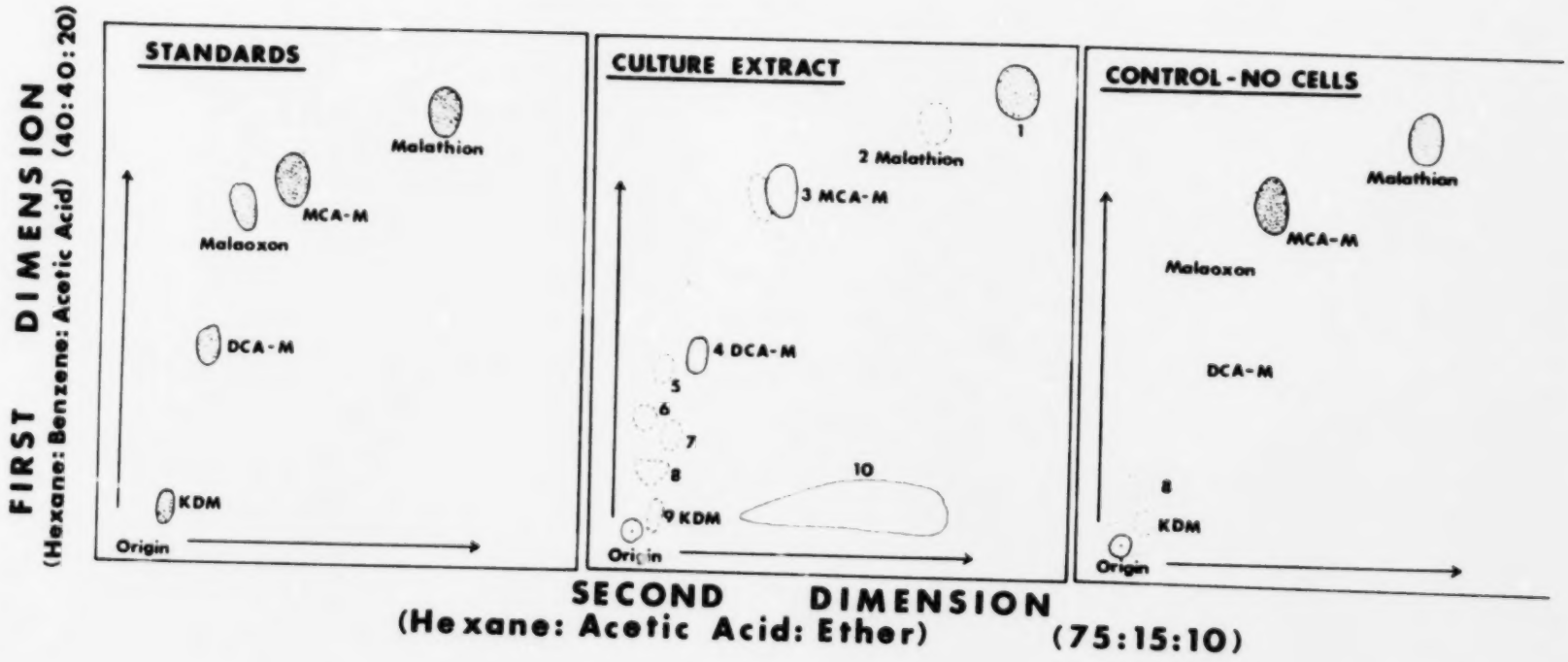


Figure 6. Thin-layer chromatogram of malathion-seawater medium with (culture extract) and without (control) an inoculum of bacterium # 45. Reference standards are included for comparison of spots.

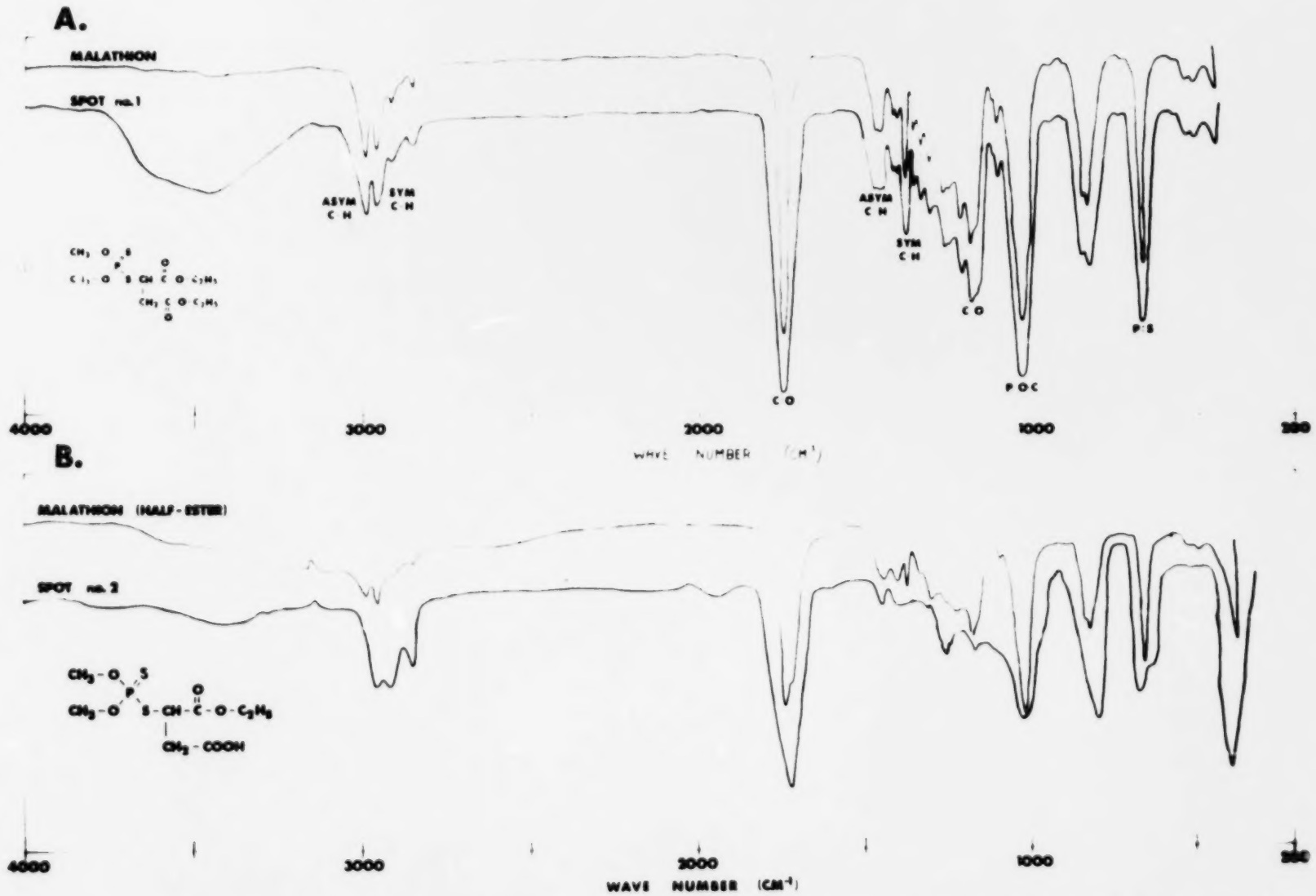


Figure 7. Infrared spectral tracings of (a) malathion and extract from band corresponding to spot 1 and (b) malathion-half-ester with extract from spot 2 (Figure 6).

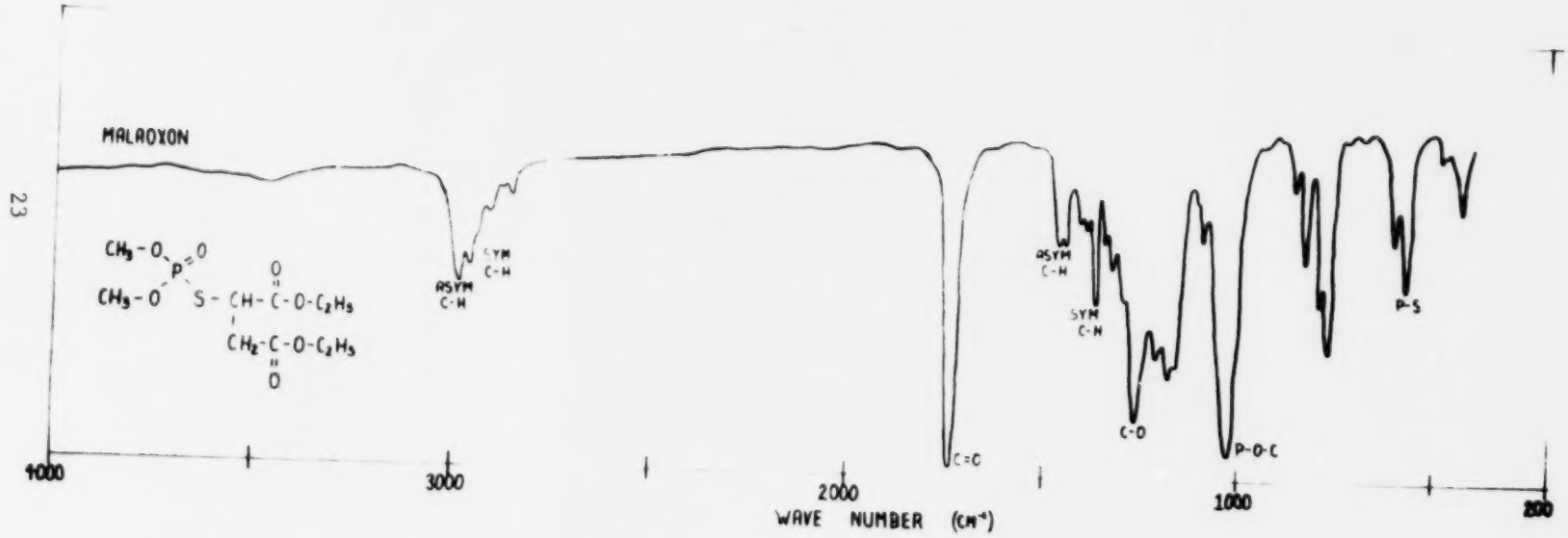


Figure 8. Infrared spectral tracing of malaoxon, with representative adsorption bands indicated.

that spot 1 is malathion. The spectral tracing of malaoxon differs in several ways from those of all other metabolites. Malaoxon (Figure 8) was not present as a metabolite in this culture fluid. The spectral tracing of TLC spot 2 is identical to that of malathion half-ester (Figure 7), and it must be concluded that spot 2 was malathion half-ester.

The spectral tracings of malathion dicarboxylic-acid and spot 3 are compared in Figure 9. All peaks are identical, indicating the spot represents that compound.

The IR spectral tracing of TLC spot 4 is illustrated in Figure 9. A comparison of this spectrum with that of standard K-desmethyl malathion indicates most peaks are accountable. The dissimilarities of the spectra are probably due to a sparcity of sample and possible water interference, as indicated by the broad band at 3400 cm^{-1}. The additional adsorption peaks located between 200-1000 cm^{-1} cannot be attributed to desmethyl-malathion. The similarity of bands in the two spectra, when combined with the thin-layer data, indicates that this metabolite is K-desmethyl malathion.

Other metabolites eluted from spots on the thin-layer plates are shown in Figures 10, 11, and 12. These compounds were found only in very low concentrations and IR spectra were obtained by using an expanded ordinate scale. Comparisons with available standards showed little similarity to these metabolites; therefore, the compounds cannot be conclusively identified.

Figure 10 shows the IR spectra of compounds from TLC Spots 5 and 6. Some major adsorption bands were identified, and from these data, a possible metabolite structure, "phosphorodithiosuccinate", was proposed for spot 5. However, it is highly probably that the sample is not pure and the compound is mercaptosuccinate, previously identified as a malathion metabolite (18). No structure is proposed for TLC spot 6.

Figure 11 shows the IR spectral tracings for compounds from TLC spots 7 and 8. Most major bands were identified, but no structure could be proposed. I believe that these samples contain phosphorous contamination, rather than the phosphorous bonds indicated on the tracings. Since the sample is in relatively low concentration, small amounts of contaminants would be greatly exaggerated by the expanded mode on the IR instrument. From previous experience (18, 20, 21), I would expect a number of thioate derivatives from phosphatase and demethylation reactions but not all phosphorothioates as the spectra indicate.

Figure 12 shows the IR spectra for compounds from TLC spots 9 and 10. Although there is less evidence for phosphorous bonds, some evidence is present (490-515 cm^{-1}). Assuming this band to be contamination,

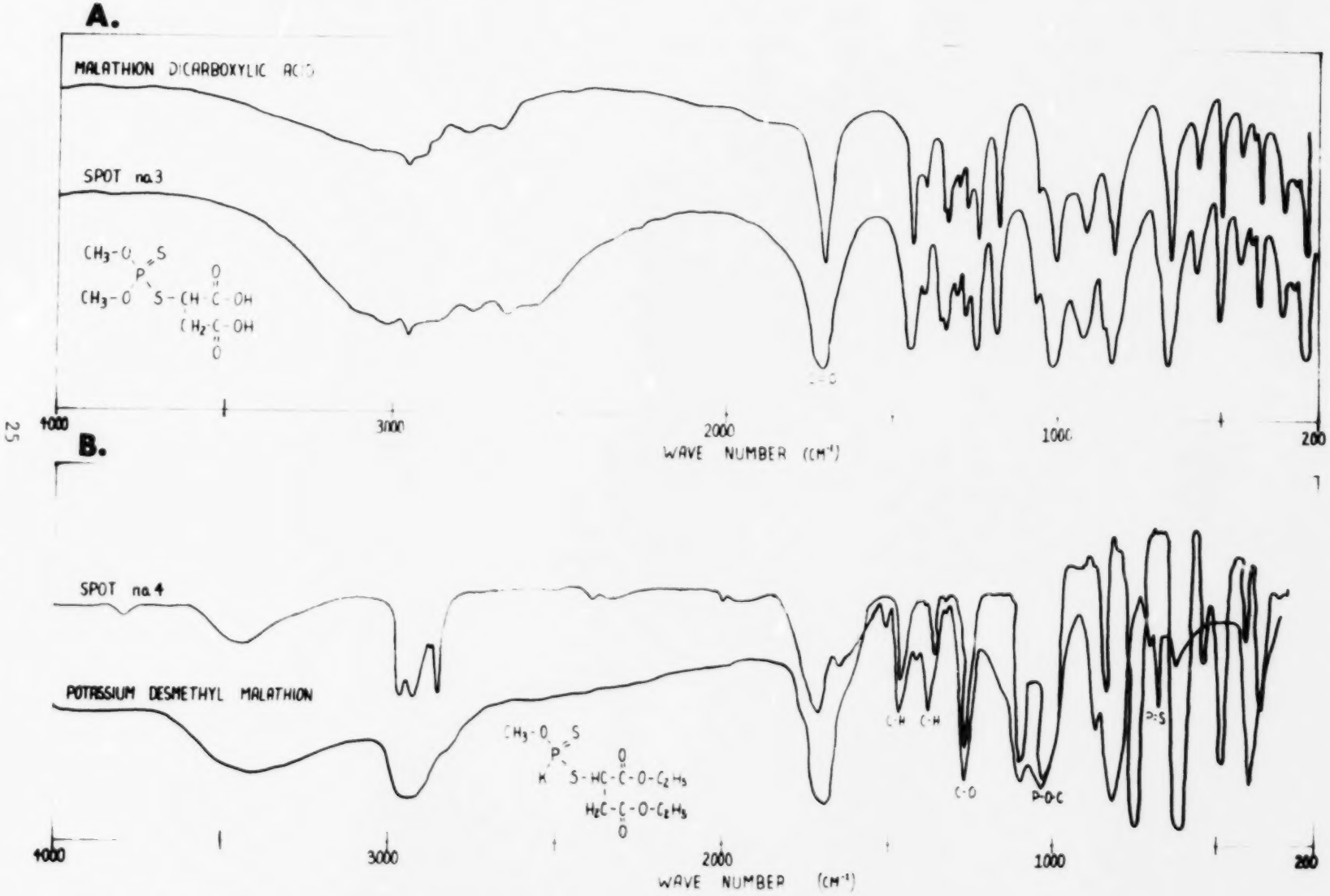


Figure 9. Infrared spectral tracings of (a) malathion-dicarboxylic acid and extract from spot 3 and (b) potassium desmethyl-malathion with extract from spot 4.

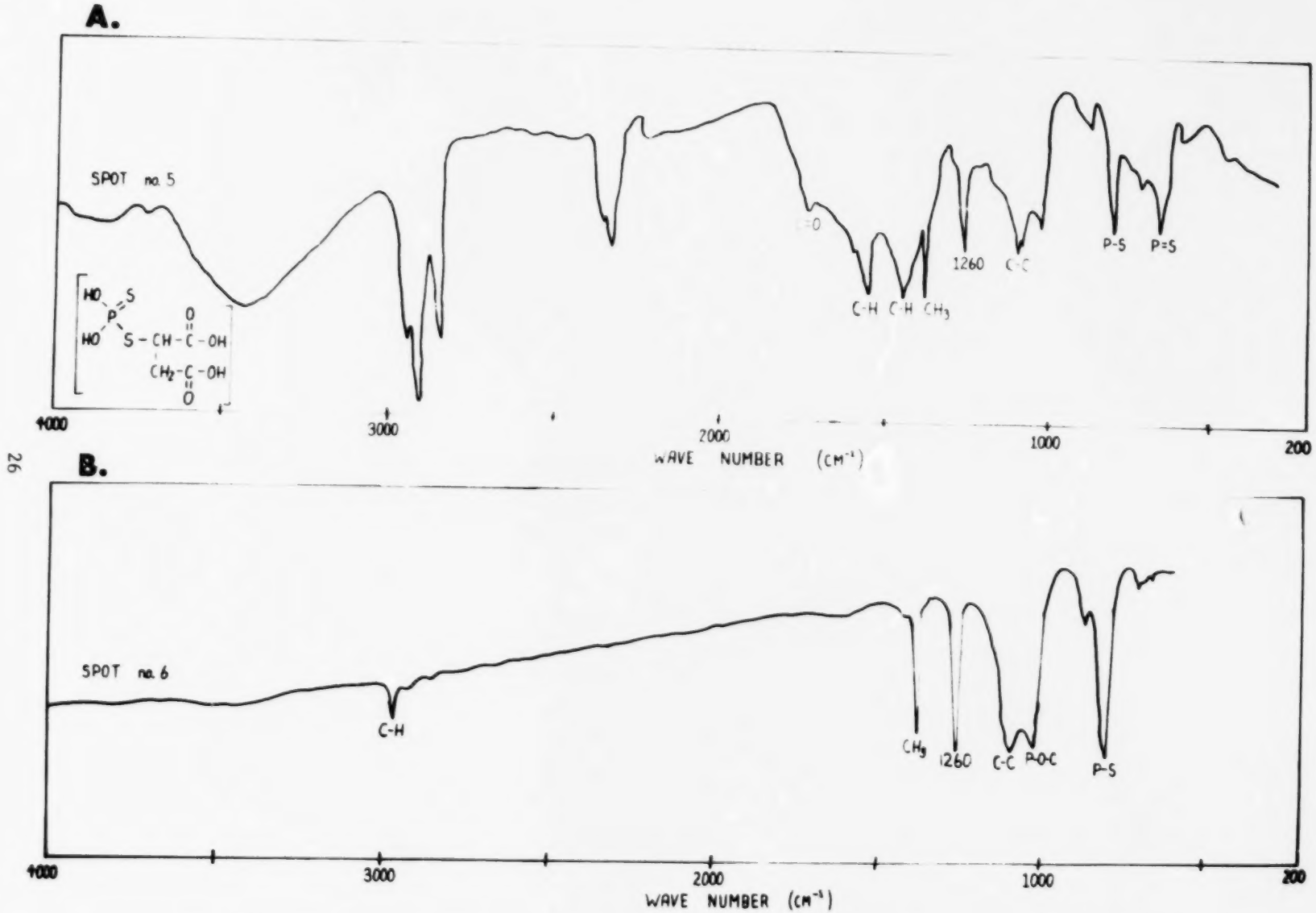


Figure 10. Infrared spectral tracings of (a) extract from spot 5 with possible structure in brackets (b) extract from spot 6.

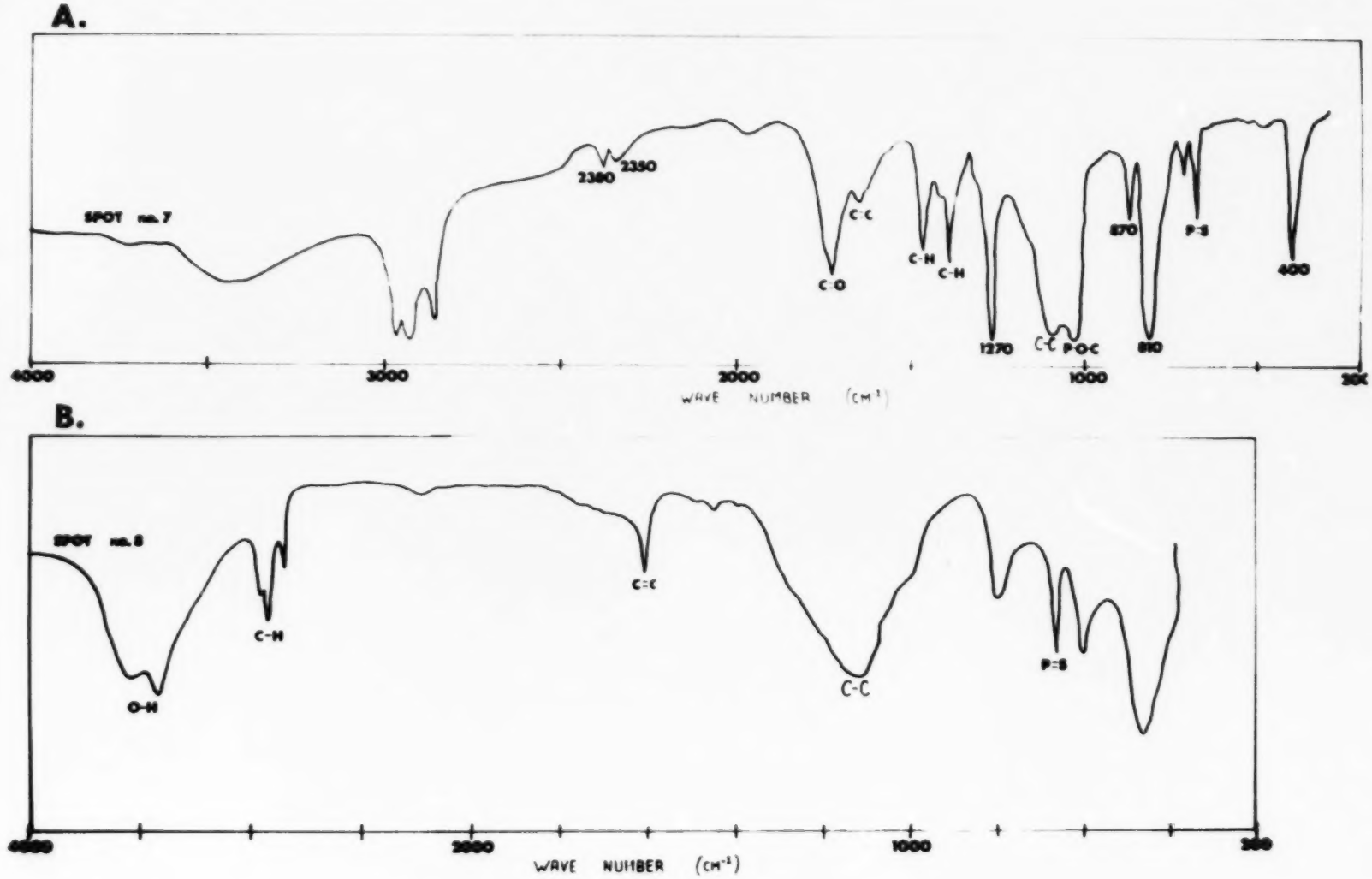


Figure 11. Infrared spectral tracings of (a) extract from spot 7 and (b) extract from spot 8.

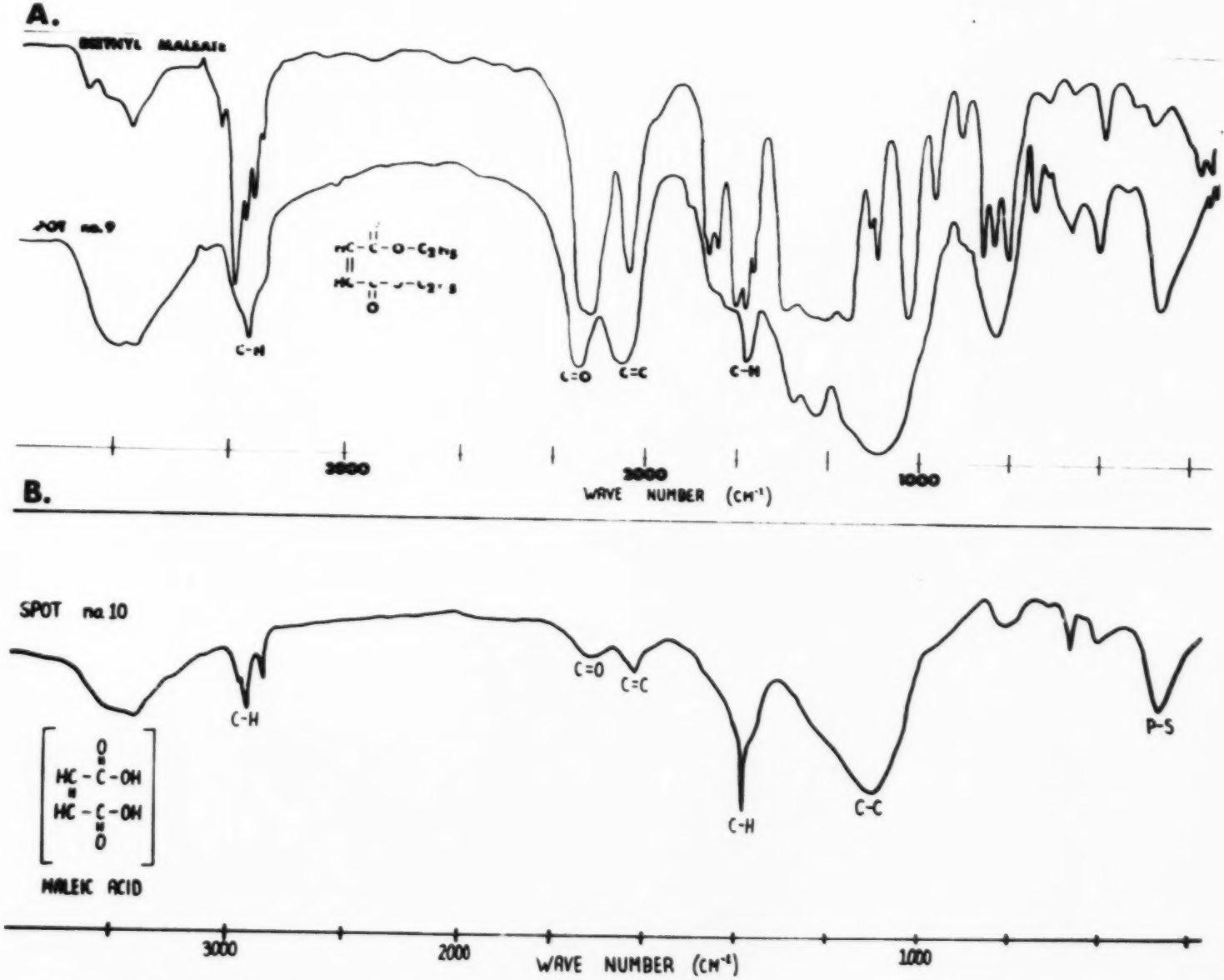


Figure 12. Infrared spectral tracings of (a) diethyl maleate and extract from spot 9 and (b) extract from spot 10, with possible structure in brackets.

there is some evidence which indicates the spots are diethyl-maleate and maleic acid, respectively. At least one of these compounds, diethyl maleate, has been identified previously as a malathion metabolite (12). The other, maleic acid, can be obtained by carboxyesterase activity from the former. No direct evidence can be obtained from these samples to identify these metabolites positively at the low concentrations found here.

Major degradation products have been identified and other metabolites tentatively identified. I believe that the low concentration of the latter metabolites is a consequence of the nature of the compound itself; most of these metabolites will serve as good carbon sources for bacteria.

ARTIFICIAL ECOSYSTEMS STUDIES

Aerobic heterotrophic bacterial numbers of 6 artificial salt-marsh environments are represented in Figures 13, 14, 15, and 16. The average results of duplicate systems that received (a) malathion, 10X field application rate (top), (b) malathion field application rate (middle), and (c) no malathion (bottom, control) are presented in Figure 13. The data represent heterotrophic counts plated in triplicate and the percentage numbers of colonies from nutrient medium which grew on replica-test medium, as compared to a representative master plate.

Essentially no differences in total heterotrophic activity (solid line) were noted between control and experimental cultures of either water (Figure 13) or sediment (Figure 14) samples. However, numbers of malathion-degrading bacteria in the treated water (Figure 13) increased over the control in water during the 30 day treatment period for both sole-carbon-degrading bacteria (SCD = 7 and 13%, dark areas) and malathion co-metabolizing bacteria (MCM = 83 and 84%, shaded area). Statistically, MCM's were significantly different($\alpha = 0.01$) among treatment levels (10X, 1X, and the control). SCD's in the water increased with treatment frequency (0, 10, 20 days; $\alpha = 0.05$) but were not significant between treatment levels. At 10X application rate only 7% of the heterotrophic bacteria on the master plate were SCD's, whereas at 1X application rate, 13% were SCD's.

Figure 14 represents similar treatment of data from the sediment samples. Fluctuations in numbers of bacteria were greater in all sediment samples but the results were similar to those of the water samples. SCD's increased significantly ($\alpha = 0.10$) among treatment levels (control, IX and 10X) and among individual treatment periods (0, 10 and 20 days; $\alpha = 0.05$), i.e., frequency of treatment. Numbers of SCD's, 4% of the heterotrophic bacteria on the master plate in the untreated sediment, were greater for 1X and 10X application rates of malathion, 8 and 10% respectively.

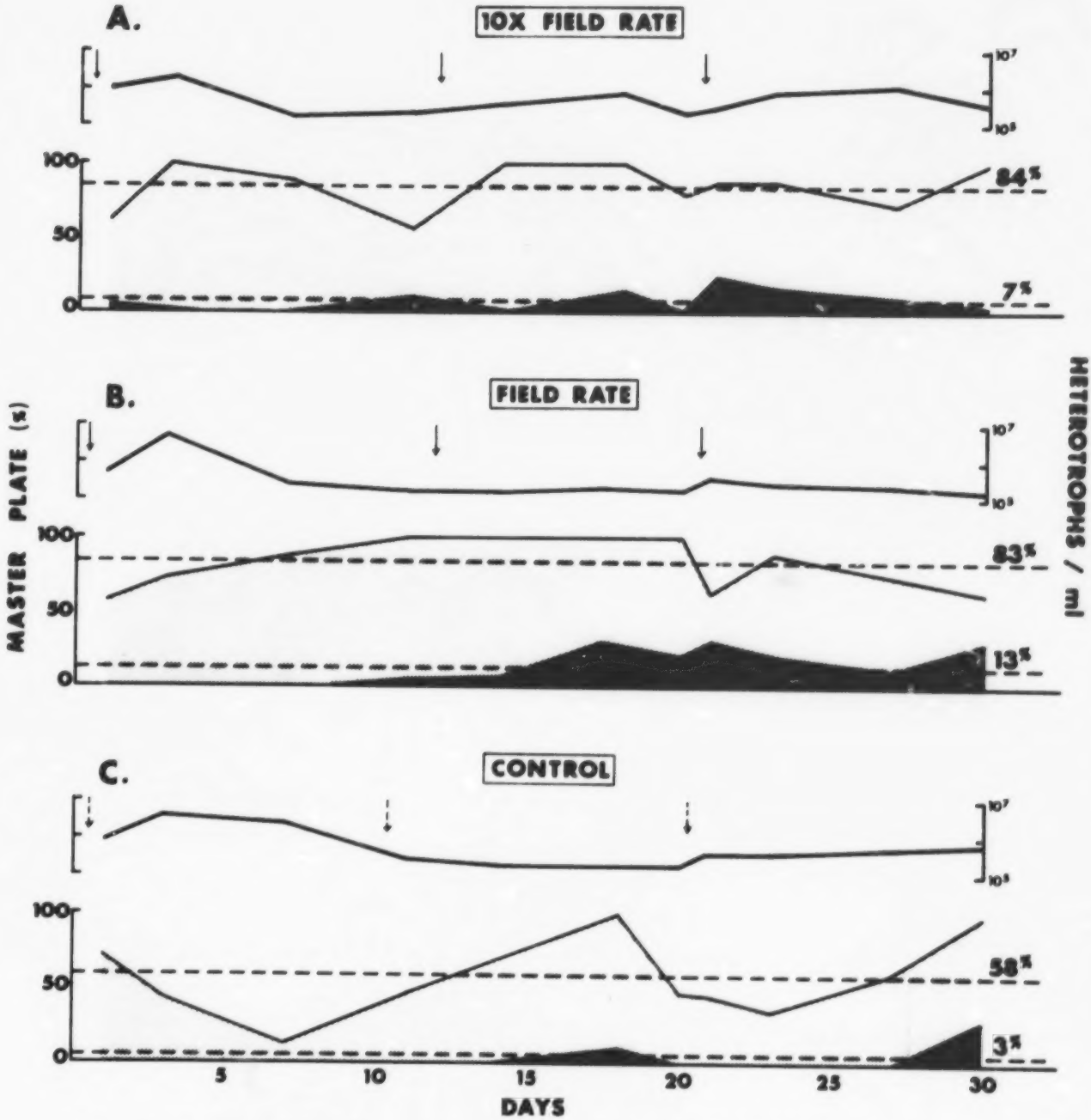


Figure 13. Heterotrophic bacteria from water column of artificial salt-marsh ecosystem presented as heterotrophs/mℓ (lin.), and number of heterotrophic bacteria which are malathion sole-carbon-degraders (dark) or malathion co-metabolizers (shaded), given as a percentage of the "master-replica-plate". Times of malathion treatment are indicated by arrows; controls are untreated. Dashed-line indicates the 30-day average for each category.

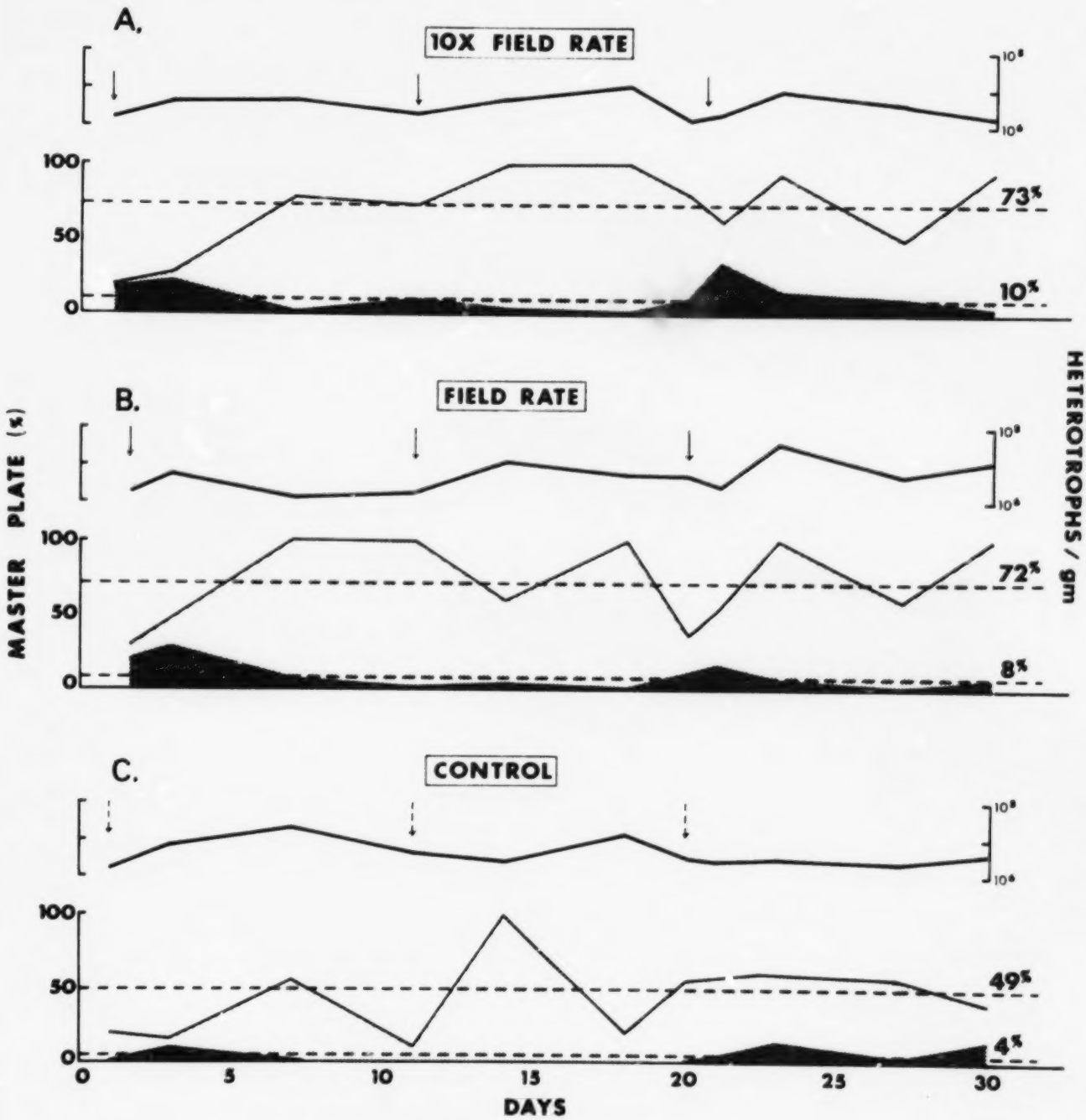


Figure 14. Heterotrophic bacteria from sediments of artificial salt-marsh ecosystems presented as heterotrophs/gm of wet sediment. Numbers of heterotrophic bacteria which are malathion sole-carbon-degraders (dark) or malathion co-metabolizer (shaded) given as a percent of the "master-replica-plate". Dashed-line indicates the 30-day average for each category. Times of malathion treatment indicated by arrows; controls are untreated.

Numbers of MCM's increased significantly with frequency of treatment (0, 10, 20 days; $\alpha = 0.1$) at both treatment levels. Although MCM bacterial numbers were not significantly different at increased levels of treatment (control, 1X and 10X; $\alpha > 0.1$); in treated vs. control systems, MCM's increased from 49% on the control to 72% and 73% of the heterotrophic bacteria on the master plate from the 1X and 10X treated systems, respectively.

Malathion increased the number of MCM's and SCD's in the water column at increased treatment levels but not with frequency of treatment. On the other hand, malathion application to artificial environments increased both MCM's and SCD's in the sediments with frequency of application levels. No significant changes in heterotrophic activities were noted with respect to amylase, lipase, chitinase, or proteinase production by the bacteria in the malathion-treated systems at either application rate, as compared to the untreated system.

To compare the effects of a recalcitrant insecticide with those of a biodegradable insecticide (malathion) on the microbial ecosystem, similar artificial environments were challenged with mirex. The data are presented in Figures 15 and 16. No differences among control and treated systems were noted for heterotrophic bacterial numbers in water or sediment samples. However, some decrease in numbers of "mirex-tolerant" bacteria (i.e., bacteria which grew on nutrient medium plus 10 µg mirex/mℓ shaded area) was noted, especially at the higher treatment level. This decrease was more pronounced after the initial treatment and appeared to recover and be unaffected by the latter treatment. No mirex-degrading bacteria were selected by the replica-plating procedure. The overall effect of mirex on the microbial ecosystem of an artificial salt-marsh environment bears little resemblance to that of the "softer" organophosphate insecticide malathion. No stimulatory or inhibitory effect, other than the initial treatment, was noted when the artificial ecosystem was treated with mirex.

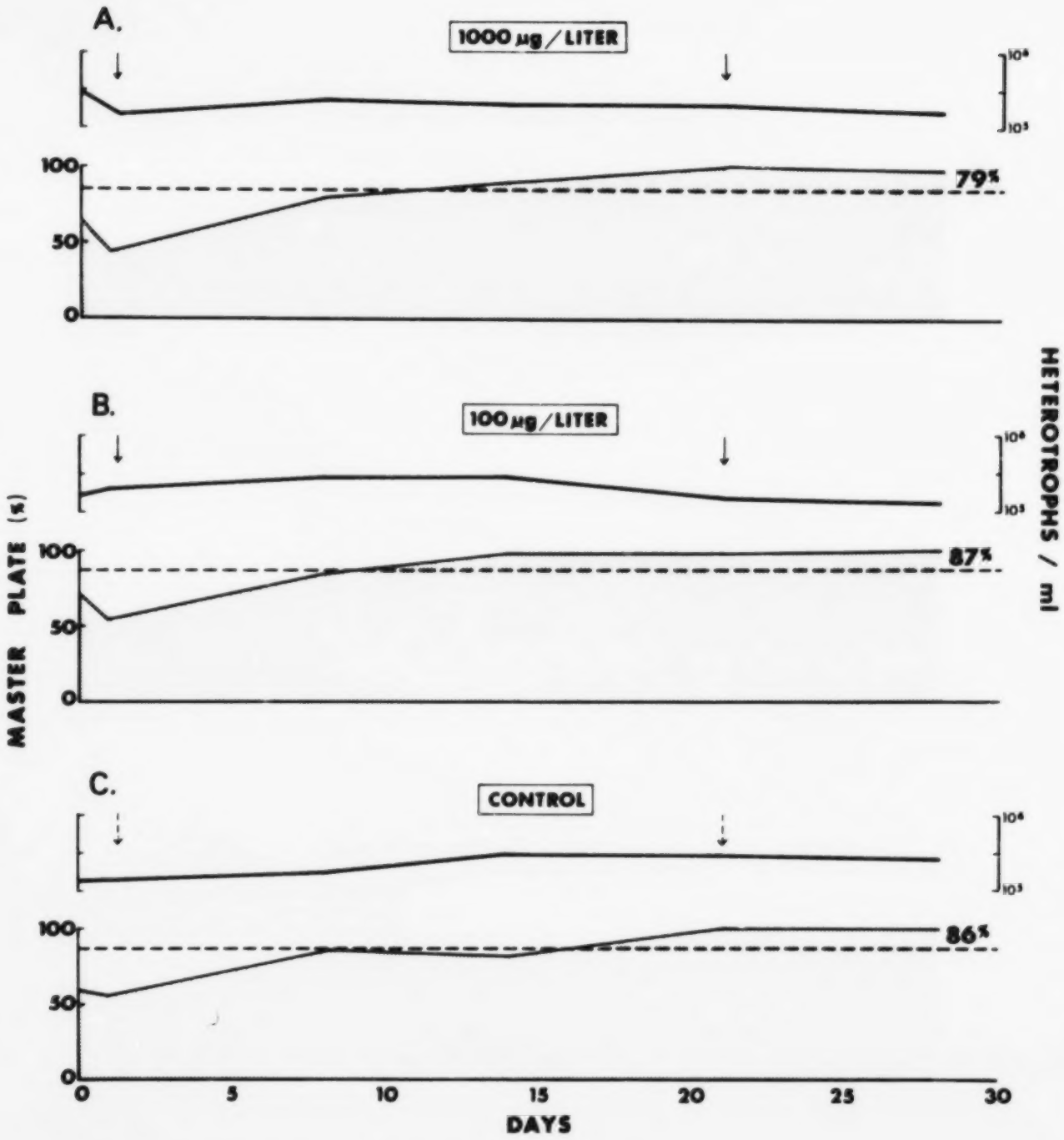


Figure 15. Heterotrophic (solid line) and mirex-tolerant bacteria (shaded) from water column of artificial salt-marsh ecosystems presented as heterotrophs/ml and percentage of "replica-master-plate", respectively.

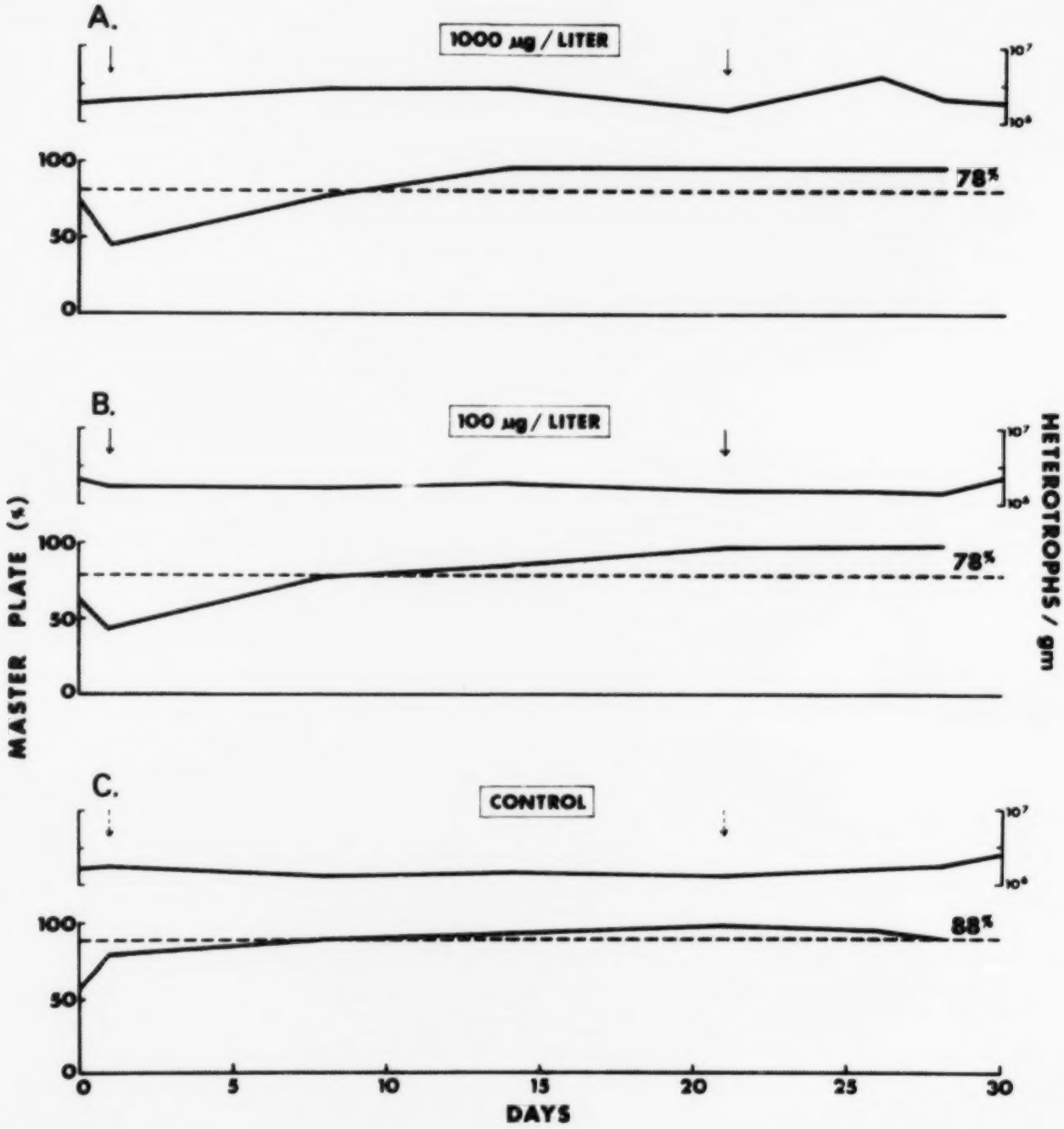


Figure 16. Heterotrophic (solid line) and mirex-tolerant bacteria (shaded) from sediments of artificial salt-marsh ecosystems.

SECTION V
DISCUSSION

Malathion, when applied to aqueous environments, was rapidly dissipated by both chemical and biological mechanisms. Like most organophosphate insecticides, malathion did not persist in aqueous environments, except under special conditions; i.e., adsorbed to Juncus grass. Even then malathion would be degraded within a short time.

Previous reports have indicated that malathion was quite stable under neutral or acid pH conditions and susceptibility to hydrolysis increased with increasing alkalinity (11, 28). Likewise, malathion is thermostable at temperatures of 21 C (70 F) and below, but rapidly dissipates at temperatures of 27-32 C (80-90 F) (11). The extent of chemical degradation *in vitro* was extensive (Figure 5), malathion having a half-life of approximately 92-96 h. I believe that chemical degradation was influenced by both temperature (27 C) and alkalinity. Malathion degradation increased with increasing alkalinity of seawater. The stability of most organophosphate insecticides in sterile seawater was a factor previously overlooked by investigators of persistence of chemicals in the estuary. Dr. W. W. Walker (personal communication Gulf Coast Research Lab., Ocean Springs, MS.) reported that a differential stability exists among organophosphates in sterile seawater environments. Further investigations into the relationship of mechanisms of chemical degradation to biological mechanisms are needed.

Although chemical degradation is important in understanding the extent and rate of malathion dissipation from the environment, biological degradation appears to be as significant in salt-marsh environments as that reported in soils (28).

We isolated 15 bacterial cultures from salt-water environments which degraded malathion *in vitro* within 10 days. Eleven cultures degraded the insecticide as a sole-carbon-source, whereas all 15 isolates degraded the compound within 5 days when an additional carbon source was added. Little malathion carbon contributed to the cell mass of the cultures tested, as evidenced by radioactive-carbon assay (Table 6). However, such contribution could have occurred only by heterotrophic CO_2-fixation or by single-carbon metabolism, because the ^{14}C-label was on the methoxy-group of the malathion molecule. Other cell-mass contribution could have been from the ethyl-group, due to carboxyesterase cleavage to form the malathion-carboxylic acids; however, the ethyl-radical was not radio-labelled and the resulting cell-mass would not have been radioactive. Most biological activity appears to be associated with an effective carboxyesterase system that causes early breakdown to the acids, with little contribution to the cell's carbon skeleton.

An understanding of the interactions of microorganisms and malathion required that we isolate and identify the principal metabolites. We employed two cultures, which effectively utilized malathion: a sole-carbon-degrader and a co-metabolizer. Both incubation mixtures containing bacterial cells yielded 10 TLC-spots which were separated by the methods described (Figure 6), as opposed to 4 or 5 spots (chemical-degradation products) produced by the uninoculated control medium. By comparison with standards, these metabolites were identified tentatively as (1) monocarboxylic acid malathion, (2) dicarboxylic acid malathion (both chemical and biological degradation products), (3) K-desmethylmalathion (small amount produced by chemical degradation), and (4) a number of compounds believed to be phosphodithionates (found only as metabolites), for which no standards were available (Table 8).

Table 8. MALATHION DEGRADATION PRODUCTS

Incubation Time	Products
Chemical Degradation:	
2 days	Malthion Monoester (MCA) Malaoxon
7 days	Malathion Diacid (DCA) Desmethyl Malathion (KDM)
Bacterial Degradation:	
2 days	MCA, DCA, KDM
10 days	Other unidentified products (Phosphothionates)

Confirmation of the identification of these metabolites was made by infrared analysis. Comparisons of the IR spectra with reference standards confirmed the identification of spot 1 as malathion (Figure 7) spot 2 as malathion monocarboxylic acid (Figure 9) spot 3 as malathion-dicarboxylic acid and 4 as O-desmethyl-malathion (potassium salt, Figure 9). Identification of some major adsorption peaks are presented and, in 2 cases, possible compound structures were presented for spots 5-10 (Figures 10, 11, and 12). However, conclusive identification could not be achieved with the small quantities of samples obtained. It should be noted, however, that detoxification (loss of ability to inhibit acetylcholinesterase) occurs only after at least 2 metabolic steps, i.e., to the dicarboxylic acid. Walker and Stojanovic (29) reported acetylcholinesterase inhibition by the monocarboxylic acid metabolite.

Malathion was rapidly degraded in vitro by salt-marsh bacteria. When applied to a simulated salt-marsh environment, malathion was degraded by the indigenous bacterial community. In a simulated environment, numbers of bacteria capable of degrading malathion in the presence of additional nutrients increased in the sediments with the increasing frequency of application, and in the water column increased, with the level of treatment (Figures 13 and 14). On the other hand, numbers of bacteria which degrade malathion as a sole-carbon-source (SCD's) were linked to the level of treatment in sediments and the frequency of treatment in the water column. In either water or sediments, SCD's did not appear to play a significant role in the degradation of malathion, comprising only about 10% of the portion of bacteria which degrade malathion. It is possible that bacteria which co-metabolize malathion (MCM's), which increased with treatments, catalyzed a more rapid degradation of the compound, resulting in decreased selection of SCD's. At the same time, the increase of SCD's in the sediment may have been a result of more malathion reaching the sediments when higher levels were applied, probably due to precipitation of the chemical to the sediments.

Malathion, when applied to salt-marsh environments, should rapidly degrade by chemical and biological mechanisms. Chemical hydrolysis is stimulated by increasing temperature and salinity, but would appear to be of lesser importance in overall degradation. Microbial degradation is more rapid than chemical degradation, and is apparently mediated by a large proportion of salt-marsh bacteria. In vitro, salt-marsh bacteria metabolize malathion to malathion half-ester, malathion dicarboxylic acid, K-desmethyl malathion, and several other unidentified metabolites. Biological degradation activity appears to be associated with carboxy-esterase activity, with delayed demethylation and with phosphatase activity. According to one previous publication (29), malathion half-ester (MCA) is the only acetylcholinesterase inhibitor among the metabolites. I expect, that, in a complex salt-marsh environment, MCA would be further degraded rapidly to other non-toxic metabolites. It appears that MCM's assume the major role in degradation of malathion and SCD's are active only when application rates are excessive. I conclude that malathion, when applied to salt-marsh environments, is rapidly degraded by the indigenous bacterial community, with no apparent adverse effect on the microbial community.

SECTION VI
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SECTION VII
ABBREVIATIONS AND SYMBOLS

C'ase	Carboxyesterase
DCA	Dicarboxylic-acid-malathion
DQC	Dibromoquinone chloride
DEE	Diethyl ether
DDT	Dichlorodiphenyltrichloroethane
EC	Effective concentration required to affect 50% of the experimental population
GLC	Gas liquid chromatography
IR	Infrared spectroscopy
KDM	Potassium desmethyl malathion
MCA	Monocarboxylic acid malathion
MCM	Malathion co-metabolizers
M. lb. A.I.	Million pounds active ingredient
nd	Not detectable
P'ase	Phosphatase
ppb	Parts per billion (µg/kg, µg/liter)
ppm	Parts per million
TLC	Thin-layer-chromatography
ULV	Ultra-low volume
°/oo	Parts per thousand